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ENZYME-HISTOCHEMICAL METHOD FOR IDENTIFICATION OF LYMPHATIC CAPILLARIES

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ABSTRACT

Using 5'-nucleotidase (5'-Nase)-alkaline phosphatase (ALPase) double staining, lymphatic capillaries and blood capillaries were distinguished histochemically on cold glycol methacrylate (JB-4) sections of monkey intestines, on the basis of both enzyme characteristics. The specificity of the 5'-Nase reaction was obtained by inhibiting nonspecific ALPase in the 5'-Nase incubation medium including L-tetramisole. This double staining method demonstrated satisfactory isolated visualization of 5'-Nase activity in lymphatic capillaries and of ALPase activity in blood capillaries under light microscopy. The intensity and localization of 5'-Nase activity on the walls of lymphatic capillaries were also determined by the lead-based method under transmission electron microscopy. The intense reaction products of 5'-Nase activity were predominantly deposited on the outer surface of the plasma membrane of the lymphatic endothelial cells.

The identification of lymphatic capillaries by conventional light microscopy is extremely difficult and generally requires establishing fine structural characteristics of lymphatic walls by electron microscopy. In early enzyme-histochemical studies, Vetter (1) and Heusermann (2) suggested that 5'-nucleotidase (5'-Nase) activity was higher in the walls of lymphatics compared with blood vessels. Recently, Werner et al (3) applied this enzymatic property to demonstrate lymphatics of the rat pharynx by light microscopy, using 5'-Nase-alkaline phosphatase (ALPase) double staining on cryostat sections. One of us (SK) (4,5) employed this double staining method along with scanning and transmission electron microscopy. We also showed (6-9) that 5'-Nase activity was useful to distinguish lymphatic capillaries from blood capillaries in various mammalian tissues including human by light and electron microscopy. In the present study, the 5'-Nase-ALPase double staining method for enzyme histochemistry with a slight modification has been applied to cold glycol methacrylate (JB-4) sections yielding excellent contrast and resolution by light microscopy. This paper introduces the new 5'-Nase-ALPase double staining method as a satisfactory technique for differentiating lymphatic capillaries from blood capillaries on plastic tissue sections.

MATERIALS AND METHODS

Experimental animals

The small intestines (ileum) of Japanese monkeys (*Macaca fuscata*) anesthetized with sodium pentobarbital (25mg/kg) were fixed by perfusion via the thoracic aorta with cold formaldehyde-$CaCl_2$ fixative (6% paraformaldehyde, 1% $CaCl_2$) in 0.1M cacodylate buffer (7% sucrose) as described previously (9,10).

Preparation of tissues for enzyme histochemistry

After perfusion, the tissues for light microscopy were excised and washed in three changes of the 0.1M cacodylate buffer. They were dehydrated, immersed in the cold glycol methacrylate (JB-4 solution A catalyzed resin, Polysciences Inc., PA, USA) and then embedded in the JB-4 medium with a hardener (JB-4 solution B) using embedding plastic molds (S-22, Polysciences, Inc., PA, USA). The polymerization of the resin was carried out at 4°C for over 8 hours. All specimens were stored in the refrigerator. Plastic sections 5-7μm thick were cut with a steel blade on a microtome and mounted on albumin coated slides. For transmission electron microscopy, 50μm sections were prepared by a Microslicer (Dosaka EM Co., Ltd., Kyoto, Japan) and also fixed in the same fixative.

5'-Nase staining for lymphatic capillaries

Light microscopy: The standard medium for the lead method of Wachstein and Meisel (11) contains: 0.2M Tris-maleate buffer (pH 7.2) 20 ml, 5'-adenosine monophosphate (AMP, sodium salt, type II, No. A-1752, Sigma) 25mg, 0.1M $MgSO_4$ 5ml, sucrose 3g, distilled water 22ml and $Pb(NO_3)_2$ (Taab) 3ml. To obtain the specific reaction for 5'-Nase activity, 20mg of L-tetramisole (T-1512, Sigma) as an inhibitor of nonspecific ALPase (final concentration 2mM) was added to the standard medium. Sections were incubated in the reaction medium for 90 min at 37°C and rinsed in distilled water, and then immersed in 1% yellow ammonium sulfide for 2 min.

Transmission electron microscopy: 50μm sections were incubated in the reaction medium for 5'-Nase activity with lead as the capture agent (12); the medium contained one-half of $Pb(NO_3)_2$ in the standard medium for light microscopy. The sections, incubated in the medium containing 2mM L-tetramisole for 30 min at 37°C, were rinsed with 0.1M cacodylate buffer. They were postfixed in 2% osmium tetroxide-0.5% potassium ferrocyanide solution in 0.1M cacodylate buffer for 1 hr at 4°C, dehydrated in ascending concentrations of ethanol and then embedded in Epol 812 (Oken, Tokyo, Japan). Ultrathin sections stained with uranyl acetate or without further staining were examined using a JEOL JEM100CX transmission electron microscope.

ALPase staining for blood capillaries

The reaction medium for ALPase activity, according to the azo dye method of Burstone (13) with a slight modification, contained: 40mg of naphthol AS-MX phosphate (disodium salt, No. N-5000, Sigma) dissolved in 2ml of NN'-dimethylformamide (No. D-4254, Sigma) and 40mg of fast blue BB (No. F-3378, Sigma) dissolved in 40ml of 0.1M Tris-buffer (pH 8.5). After incubation for 5'-Nase activity, the sections were further incubated in the reaction medium for 2 hrs at 4°C. After incubation, each section was thoroughly rinsed in distilled water and mounted in glycerin.

Control experiments for 5'-Nase and ALPase staining

The reaction medium was altered as follows: 1) substrate (AMP or naphthol AS-MX phosphate 50mM with $NiCl_2$ or 5mM L-tetramisole in final concentration before being incubated in the reaction medium; 2) specimen was incubated at 60°C for 60 min before incubation.

RESULTS AND DISCUSSION

The product of 5'-Nase activity was clearly detected as a dark brown precipitate of lead sulfide on the walls of lymphatic capillaries on the plastic section incubated in the reaction medium containing L-tetramisole whereas little or no activity was seen in blood capillaries (*Fig. 1A*). When the sections were then incubated to demonstrate ALPase activity, the blood capillaries were visualized in blue (*Fig. 1B*). With the 5'-Nase-ALPase double staining no change in intensity or localiza-

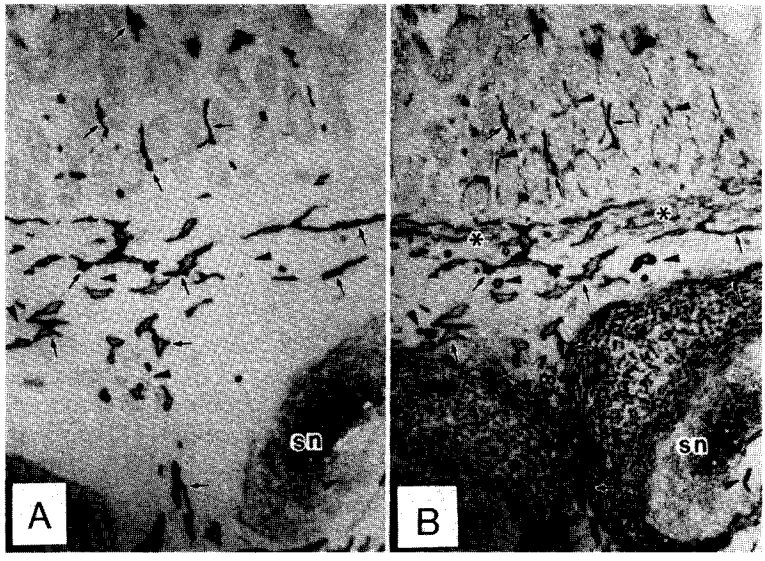

Fig. 1. Light micrographs of a JB-4 section of the ileum near ileocecal junction of monkey with histochemical demonstration of lymphatic capillaries and blood capillaries. ***A****: 5'-Nase activity. Dark staining reveals lymphatic capillaries (arrows) with irregularly shaped spaces in the lamina propria and tela submucosa. The blood capillaries (arrowheads) reveal no 5'-Nase activity.* ***B****: 5'-Nase and ALPase activity of the same section as in* Fig. 1**A**. *The blood capillaries (arrowheads) are now demonstrated by virtue of ALPase activity. Asterisks show lamina muscularis mucosae. sn.: secondary nodule. 150x.*

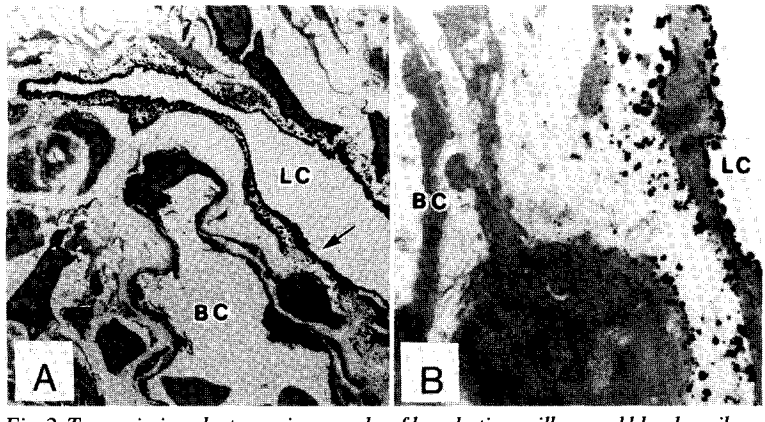

Fig. 2. Transmission electron micrographs of lymphatic capillary and blood capillary in the tela submucosa of another specimen incubated in the medium with L-tetramisole. ***A****: Note the 5'-Nase-positive lymphatic capillary (LC) and the 5'-Nase-negative blood capillary (BC). 2300x.* ***B****: Further magnification of both capillary walls shown in* Fig. 2**A** *(arrow). Dense granular precipitates are seen on the outer surface of the endothelial cell membranes and also in the area on the basal side. 10,000x.*

tion of the 5'-Nase staining compared to that of the 5'-Nase above was seen. ALPase staining did not change either, regardless of whether or not the section had previously been stained to demonstrate 5'-Nase activity. However, when ALPase staining was followed by the 5'-Nase procedure, a comparatively weak reaction of 5'-Nase activity was obtained in the lymphatic capillaries on plastic sections as reported previously on the cryostat sections (4-6).

In our previous studies (4-8) of 5'-Nase-ALPase double staining on the cryostat sections, the incubation mixture did not contain L-tetramisole when demonstration of 5'-Nase activity was followed by the ALPase reaction. Because in addition to 5'-Nase, nonspecific ALPase also metabolizes AMP (14), ALPase activity is found in both lymphatic and blood capillaries with incubation using the standard medium of Wachstein and Meisel (11). Thus, the walls of blood capillaries are stained less deeply blue with fast blue BB because the brown precipitate of nonspecific ALPase reacts in the 5'-Nase procedure. To obtain specific demonstration of 5'-Nase activity, complete inhibition of ALPase activity by L-tetramisole is required. L-teramisole is effective for this purpose because it inhibits ALPase activity without affecting 5'-Nase activity (15). Control sections incubated in substrate-free medium or treated for 60 min at 60C or with $NiCl_2$ before being incubated in the complete reaction medium, also showed no staining. In this way, specific 5'-Nase activity in the lymphatic capillaries has been successfully demonstrated. In regards to structural integrity, a plastic section is superior to a frozen section and it also preserves phosphatases. Therefore, this 5'-Nase-ALPase double staining method permits satisfactory differentiation of lymphatic capillaries from blood capillaries.

Using transmission electron microscopy, the lead-containing reaction products of 5'-Nase activity can be localized on the surface of the lymphatic endothelial cells but not in the blood capillaries (*Fig. 2*). By combining observations of enzyme histochemistry by light and electron microscopy, more accurate identification and demonstration of fine distribution and three-dimensional architecture of lymphatics and blood capillaries is now possible (6,9,10).

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